

Distinct Forebrain and Cerebellar Isozymes of Type II Ca^{2+} /Calmodulin-dependent Protein Kinase Associate Differently with the Postsynaptic Density Fraction*

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Forebrain and cerebellar Type II Ca^{2+} /calmodulin-dependent protein kinases have different subunit compositions. The forebrain holoenzyme, characterized in our laboratory, is a 650-kDa holoenzyme composed of 50-kDa α -subunits and 60-kDa β -subunits assembled in approximately a 3:1 ratio (Bennett, M. K., Erond, N. E., and Kennedy, M. B. (1983) *J. Biol. Chem.* 258, 12735-12744). The cerebellar isozyme is a 500-kDa holoenzyme composed of α -subunits and β -subunits assembled in almost the converse ratio, approximately four β -subunits for each α -subunit. When compared by tryptic peptide mapping and by immunochemical techniques, the β -subunits from the two brain regions are indistinguishable and the α -subunits appear closely related.

The specific activities, substrate specificities, and catalytic constants of the cerebellar and forebrain isozymes are similar, suggesting that the α - and β -subunits contain similar catalytic sites. However, two differences in the properties of the isozymes may result in functional differences between them *in vivo*. First, the apparent affinity of the cerebellar kinase for Ca^{2+} /calmodulin is 2-fold higher than that of the forebrain kinase. Second, the two isozymes appear to associate differently with subcellular structures. Approximately 85% of the cerebellar kinase and 50% of the forebrain kinase remain in the particulate fraction after homogenization under standard conditions. However, they are present in different amounts in postsynaptic density fractions. Postsynaptic densities prepared from forebrain contain the forebrain isozyme. Immunochemical measurements show that it comprises ~16% of their total protein. In contrast, postsynaptic densities prepared from cerebellum contain the cerebellar isozyme, but it comprises only ~1-2% of their total protein. Thus, the α -subunit may play a role in anchoring Type II Ca^{2+} /calmodulin-dependent protein kinase to postsynaptic densities.

Many agents regulate the functions of target cells by altering the concentration of an intracellular second messenger such as a cyclic nucleotide, Ca^{2+} ion, or diacylglycerol (1-4). The cyclic nucleotides and diacylglycerol are believed to act

primarily through the activation of specific protein kinases (5-7). The actions of Ca^{2+} as a second messenger are more diverse and often require the Ca^{2+} -binding protein calmodulin (8). Among the enzymes regulated by Ca^{2+} /calmodulin are a family of protein kinases. Ca^{2+} /calmodulin-dependent protein kinase activities have been described in mammalian brain (9, 10), pituitary (11), liver (12), pancreas (13, 14), skeletal and smooth muscle (15-18), avian erythrocytes (19), *Torpedo* electric organ (20), and *Aplysia* ganglia (21, 22). Recent structural characterization of some of these kinases suggests that there are at least four and perhaps more distinct Ca^{2+} /calmodulin-dependent protein kinases (15-18, 23-32). The diversity of these kinases may underlie, in part, the diversity of cellular responses to Ca^{2+} .

We recently reported the purification and characterization of a Ca^{2+} /calmodulin-dependent protein kinase with a broad substrate specificity that is far more highly expressed in brain than in other tissues (27). Brain kinases that are structurally similar and may be identical with this one have been described by other groups (28, 29, 31, 32). Two of the groups have referred to the kinase as " Ca^{2+} /calmodulin-dependent kinase II" (29, 31). Because this kinase now appears to occur as a family of homologous but distinct forms in different tissues as well as brain regions (29, 33, 34, and this report), we refer to them as "Type II" Ca^{2+} /calmodulin-dependent protein kinases. These kinases have several features in common. They are multimeric proteins of high molecular mass (300-700 kDa), composed of structurally related 50-60-kDa subunits. They exhibit similar substrate specificities. All the kinase subunits bind calmodulin and undergo a Ca^{2+} /calmodulin-dependent autophosphorylation. The kinases differ in the exact molecular weights and ratios of their subunits.

The brain Type II CaM kinase¹ previously described by this lab (27) is a 650-kDa holoenzyme composed of ~9 α (50 kDa)- and ~3 β/β' (60/58 kDa)-subunits. This isozyme of the kinase is concentrated in the forebrain (cortex and hippocampus) and makes up about 75% of the total brain Type II CaM kinase.² In this paper we report the purification of a distinct isozyme of the kinase from cerebellum. It is composed of subunits similar to those of the forebrain kinase, but they are assembled in a different ratio. We compare its structural and enzymatic properties to those of the forebrain kinase.

We and others have shown that the Type II CaM kinase is a major constituent of brain postsynaptic density fractions (36-38). The postsynaptic density is a specialization of the submembranous cytoskeleton that occurs beneath postsyn-

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¹ For brevity, we will frequently refer to the brain Type II Ca^{2+} /calmodulin-dependent protein kinases as Type II CaM kinases. They are not related to the Type II cAMP-dependent protein kinase.

² N. Erond, M. Bennett, and M. Kennedy, unpublished observations.

aptic membranes in CNS neurons. We show here that forebrain PSD³ fractions contain the forebrain isozyme as a major component while cerebellar PSD fractions contain the cerebellar isozyme, but in greatly reduced amounts. A preliminary report of part of this work has appeared (33). After this manuscript was submitted, another paper describing the physical properties of the cerebellar isozyme was published (34).

EXPERIMENTAL PROCEDURES

Materials—Alcohol dehydrogenase, ATP, bovine serum albumin, carbonic anhydrase, casein, chloramine-T, EDTA, EGTA, fast green, fibrinogen, hemoglobin (Type II), histones (Type VIII-S), imidazole, ovalbumin, phosvitin, PMSF, polyinosinic-polycytidylic acid, and soybean trypsin inhibitor were purchased from Sigma. Aldolase, blue dextran 2000, catalase, ferritin, protein A, Sepharose 4B, and thyroglobulin were purchased from Pharmacia. DEAE-Bio-Gel A, SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Nitrocellulose membranes (BA85, 0.45 μm) were purchased from Schleicher and Schuell, cellulose-coated thin layer chromatography plates from Eastman, trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone from Worthington, ultrapure sucrose from Bethesda Research Laboratories, and Coomassie Brilliant Blue from Searle Diagnostic. Sodium perchlorate was purchased from G. Frederick Smith Chemical Co., dithiothreitol from Boehringer Mannheim, leupeptin from Peninsula Laboratories, Na^{125}I (carrier-free) from Amersham, and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ from ICN Nutritional Biochemicals. Simonsen albino rats (140–160 g, male) were purchased from Simonsen Laboratories (Gilroy, CA). Immunoprecipitin was purchased from Bethesda Research Laboratories and washed as described by Kessler (39).

Rabbit anti-mouse IgG antiserum was prepared as described previously (36). ^{125}I -labeled protein A (2–3 Ci/mmol) was prepared by the chloramine-T method (40). Synapsin I was prepared by a modification of the method of Ueda and Greengard (41) as described previously (27). Calmodulin was purified from bovine brain by the method of Watterson *et al.* (42). Microtubule protein was purified by the method of Shelanski *et al.* (43). MAP₂, purified from microtubule protein by the method of Kim *et al.* (44), was the kind gift of James Soha of the California Institute of Technology. Calmodulin-Sepharose was prepared by the method of March *et al.* (45).

Purification of the Cerebellar Type II Ca^{2+} /Calmodulin-dependent Protein Kinase—The purification was carried out as previously described (27) with several modifications. All operations were carried out at 4 °C.

Brains were removed from 90 rats after stunning and decapitation. The cerebellar lobes, weighing approximately 0.23 g each, were removed and immediately homogenized by 12 up and down strokes in a Teflon/glass homogenizer driven at 900 rpm in 10 volumes of Buffer A (40 mM Tris (pH 7.5), 1 mM imidazole, 150 mM Na^+ -perchlorate, 250 mM sucrose, 1 mM EGTA, 5 mM EDTA, 2 mM dithiothreitol, 0.1 mM PMSF, 1 mg/l of leupeptin, and 25 mg/l of soybean trypsin inhibitor). The crude homogenate was centrifuged at $2000 \times g$ for 20 min and the resulting supernatant was centrifuged at $170,000 \times g$ for 1 h.

The $170,000 \times g$ supernatant was diluted 5-fold into Buffer B (20 mM Tris (pH 7.5), 1 mM imidazole, 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN_3), mixed with 50 ml of DEAE-agarose previously equilibrated with Buffer B, and stirred slowly for 90 min. The gel slurry was poured into a Buchner funnel and excess supernatant was drawn off by gentle suction. The resulting gel slurry was poured into a glass column (1.5 \times 30 cm) and packed at 80–100 ml/h. The column was washed with 100 ml of Buffer B containing 0.01 M NaCl. Enzyme activity was eluted with a linear gradient of 0.01–0.30 M NaCl in Buffer B (total volume of 500 ml) at 80 ml/h while collecting 6-ml fractions. Kinase activity peaked at 0.08 M NaCl. Peak fractions were pooled, adjusted to 0.1 M Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF, and brought to 50% saturation by the slow addition of solid ammonium sulfate over 1 h. After 3–12 h, precipitated protein was collected by centrifugation and redissolved

in 5 ml of Buffer C (40 mM Tris (pH 7.5), 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN_3).

Equal volumes of the redissolved ammonium sulfate precipitate were layered onto each of four 37-ml linear gradients of 5–20% sucrose in Buffer C. The gradients were centrifuged for 24 h at $27,000 \times g$ in a Beckman SW 27Ti rotor. One-ml fractions were collected with an Isco Model 184 density gradient fractionator while monitoring absorbance at 280 nm. Kinase activity appeared approximately midway through the gradient.

For determination of the Stokes radius, gel filtration through Sepharose 4B replaced the sucrose density gradient centrifugation step. The ammonium sulfate precipitate was redissolved in 4 ml of Buffer C, adjusted to 20% (v/v) glycerol, and loaded on a Sepharose 4B column (1.25 \times 100 cm) equilibrated with Buffer C plus 20% glycerol. The column was run at a flow rate of 3.2 ml/h while collecting 0.6-ml fractions. Kinase activity eluted as a single peak at approximately 80 ml.

The sucrose gradient (or Sepharose 4B gel filtration) fractions containing kinase activity were pooled and adjusted to 0.1 M Tris (pH 7.5), 0.2 M NaCl, 2 mM dithiothreitol, 0.1 mM PMSF, 2.5 mg/l of leupeptin, and 0.4 mM free Ca^{2+} . This pool was then applied to a calmodulin-Sepharose affinity column (0.9 \times 2.5 cm) equilibrated with Buffer D (40 mM Tris (pH 7.5), 0.4 mM CaCl_2 , 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN_3) containing 0.2 M NaCl. The column was washed with 4 column volumes of Buffer D containing 2.0 M NaCl, 5 column volumes of Buffer D, and 3 column volumes of Buffer C. Enzyme activity was then eluted with a linear gradient (12 ml total volume) of 0–0.2 M NaCl in Buffer C while collecting 0.5-ml fractions. Kinase activity eluted at approximately 0.08 M NaCl.

The forebrain Type II CaM kinase was purified from whole brain or from forebrain as described by Bennett *et al.* (27) except that homogenization and batch DEAE-absorption were performed as described above. This resulted in a higher recovery of forebrain kinase (3–5 mg/120 g wet weight of brain) than previously reported.

Preparation of the Postsynaptic Density Fraction—Postsynaptic density fractions were prepared using Triton X-100 as described by Carlin *et al.* (46). Briefly, brains were removed from 25 rats and the cortices and cerebella were separated and homogenized in isotonic sucrose. A mixed synaptosomal/mitochondrial pellet was obtained by differential centrifugation and then fractionated by centrifugation through a discontinuous sucrose gradient. The synaptosomal fraction obtained from the 1.0:1.3 M sucrose interface was adjusted to 0.5% Triton X-100, 6 mM Tris, pH 8.1, and insoluble material was pelleted by centrifugation. This material was resuspended and centrifuged through a second discontinuous sucrose gradient. The material at the interface of the 1.5 and 2.0 M sucrose steps, which is enriched in PSDs, was resuspended in 0.5% Triton X-100, 75 mM KCl and then pelleted by centrifugation. The final pellet was resuspended in 40 mM Tris, pH 8.0, and used for further characterization. Yields were approximately 0.6–0.7 mg/10 g wet tissue from cerebellum and 2–3 mg/10 g wet tissue from forebrain.

Protein Phosphorylation Assays—Kinase activity was assayed as described (27) except that 10 mM dithiothreitol was included in the final reaction mixture. Autophosphorylation of kinase subunits and endogenous phosphorylation of PSDs was performed as described (27) except that $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was used at a lower concentration (5–10 μM) and higher specific activity ($2.0\text{--}5.0 \times 10^4$ cpm/pmol) and an additional 6 μl of β -mercaptoethanol was added to the stopped reactions just prior to gel electrophoresis.

Determination of Catalytic Constants—Kinetic parameters were measured as described above except that the amount of Synapsin I was increased to 20 μg , except when it was the variable, and the reaction time was decreased to 10–15 s to ensure measurement of initial rates. Each tube contained 20–50 ng of purified enzyme (4–10 nM subunits). Utilization of substrates (ATP and Synapsin I) was less than 10% in each assay. The apparent K_m values of various substrates and the apparent K_a for Ca^{2+} /calmodulin were determined by fitting the data to the Michaelis-Menton equation with a weighted nonlinear least squares computer program adapted from Cleland (47). The apparent K_m for ATP and the apparent K_a values for Ca^{2+} /calmodulin were compared in duplicate experiments using the same reagents.

Preparation of Antibodies—Monoclonal antibodies 6G9 and 4A4 were produced and selected as described (35, 36). These antibodies were purified from ascites fluids by precipitation with 50% ammonium sulfate followed by chromatography on Protein A-Sepharose or by two successive 50% ammonium sulfate precipitations. Polyclonal

³ The abbreviations used are: PSD, postsynaptic density fraction; PMSF, phenylmethylsulfonyl fluoride; MAP₂, microtubule-associated protein 2; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid.

rabbit antisera were produced by multiple subcutaneous injections of purified forebrain Type II kinase or of electrophoretically purified subunits in phosphate-buffered saline containing an equal weight of polyinosinic-polycytidylic acid. The antisera were the kind gifts of Ngozi Erundu and Mark Bennett of this laboratory.

Immunoblots and Radioimmunoassays—Proteins were separated by SDS-PAGE and transferred to nitrocellulose paper as described by Towbin *et al.* (48). The nitrocellulose sheets containing transferred protein were labeled with antibodies as previously described (36). Radioimmunoassay was carried out by a quantitative immunoblot method, as described by Erundu and Kennedy (35) using either monoclonal antibody 6G9 for detection of the α -subunit or a rabbit polyclonal antiserum for detection of the β -subunit. Briefly, standard amounts of purified forebrain Type II Ca^{2+} /calmodulin-dependent protein kinase and unknown samples were separated by SDS-PAGE and transferred to nitrocellulose paper as above. The nitrocellulose sheets were labeled with appropriate antibodies and subjected to autoradiography after drying. The labeled bands were located on the nitrocellulose sheet using the autoradiograph, cut out, and counted in a γ counter. A standard curve was constructed from the counts bound to standard amounts of forebrain Type II CaM kinase (after subtracting background). Linear standard curves were obtained on log/log plots for 20–500 ng of α -subunit with 6G9 and for 80–500 ng of β -subunit for the rabbit antiserum.

Other Procedures—Protein concentrations were measured by the method of Lowry *et al.* (49) as modified by Peterson (50). SDS-PAGE was performed by the method of Laemmli (51). Stacking gels ($2.5 \times 16 \text{ cm} \times 1.5 \text{ mm}$) contained 3.5% acrylamide, 0.09% bisacrylamide and the running gels ($14 \times 16 \text{ cm} \times 1.5 \text{ mm}$) contained 10% acrylamide, 0.27% bisacrylamide. Molecular weight standards for SDS-PAGE were: MAP₂ (300,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), ovalbumin (43,000), alcohol dehydrogenase (41,000), carbonic anhydrase (29,000), chymotrypsinogen (25,000), and soybean trypsin inhibitor (21,500). The sedimentation coefficient ($s_{20,w}$) of the kinase was determined by sucrose density gradient centrifugation as described by Martin and Ames (52) with ovalbumin ($s_{20,w} = 3.5 \text{ S}$), fibrinogen ($s_{20,w} = 7.9 \text{ S}$), catalase ($s_{20,w} = 11.3 \text{ S}$), and thyroglobulin ($s_{20,w} = 19.2 \text{ S}$) as standards. The Stokes radius (\AA) of the kinase was determined by gel filtration through Sepharose 4B as described by Porath (53), with catalase (52 \AA), ferritin (61 \AA), thyroglobulin (85 \AA), and fibrinogen (107 \AA) as standards. Iodinated tryptic maps were prepared by the method of Elder *et al.* (54).

RESULTS

Purification of Cerebellar Type II Ca^{2+} /Calmodulin-dependent Protein Kinase

Purification—The homogenization conditions described in our initial report on the purification of brain Type II CaM kinase (27) have been altered to optimize recovery of the cerebellar kinase. When the cerebellum was homogenized in the original homogenization buffer, 80–90% of the Type II CaM kinase activity was recovered in the particulate fraction after centrifugation. The addition of the chaotropic salt, sodium perchlorate (0.15 M), to the homogenization buffer released most of the bound kinase resulting in the recovery of 70–80% of it in a soluble form. The cerebellar kinase activity was unstable in the presence of calcium, therefore 0.2 mM CaCl_2 was replaced by 1 mM EGTA, 5 mM EDTA. The solubilized enzyme was adsorbed to DEAE-agarose by a batch process because of the large sample volume after dilution of the $170,000 \times g$ supernatant to an appropriate ionic strength. The purification of the cerebellar kinase is summarized in Table I.

Different salt concentrations were required to elute the forebrain and cerebellar Type II CaM kinases from calmodulin-Sepharose. The forebrain isozyme was eluted in EGTA buffers without salt (27), while elution of the cerebellar isozyme required both EGTA and $\sim 0.08 \text{ M}$ NaCl. This may result from nonspecific ionic interactions of the cerebellar kinase with the column or from specific low affinity binding of the kinase to calmodulin even in the absence of Ca^{2+} . The

calmodulin-Sepharose pool was approximately 90% pure as judged by densitometric scans of stained SDS-polyacrylamide gels. It represented a purification of about 400-fold from the crude homogenate with a yield of approximately 5%. The low overall recovery was due largely to low recoveries from DEAE-agarose. The specific activities of the calmodulin-Sepharose pools varied from 1.23 to 2.90 $\mu\text{mol}/\text{min}/\text{mg}$ under our standard assay conditions and from 3.4 to 8.0 $\mu\text{mol}/\text{min}/\text{mg}$ at saturating synapsin I concentrations. This compares to an average value for the forebrain kinase of 2.9 $\mu\text{mol}/\text{min}/\text{mg}$ under standard conditions and 8.0 $\mu\text{mol}/\text{min}/\text{mg}$ at saturating synapsin I concentrations. The physical properties of the forebrain kinase were not altered by the changes in purification procedure and recoveries were improved, ranging from 6 to 11%.

Subunit Composition—The Type II CaM kinases purified from cerebellum and from forebrain both contain two major subunits, α (50 kDa) and β (60 kDa), but they are present in different ratios (Fig. 1). Minor subunits of 56 and 58 kDa (β') in the cerebellar kinase are more prominent than the 58-kDa β' -subunit in the forebrain kinase. As for the forebrain kinase, the cerebellar β - and β' -subunits have identical peptide maps and occur in a constant ratio to the α -subunits from preparation to preparation. All of the subunits of the cerebellar kinase were precipitated by a monoclonal antibody (4A4) that recognizes only the α -subunit on Western blots (data not shown). Thus, they exist together in a holoenzyme complex. In several immunoprecipitation experiments with antibody 4A4, 6–7% of the initial kinase activity remained in the supernatant after immunoprecipitation with maximal amounts of antibody (data not shown). Thus, a small proportion of the cerebellar kinase holoenzyme molecules may not contain α -subunits.

The physical properties of the cerebellar isozyme were determined by the methods used to characterize the forebrain kinase (27). The molar ratio of the subunits was determined from densitometric scans of gels stained with fast green. The β' -doublet was treated as a single band for this analysis. The ratio of β -subunit alone to α -subunit was 3:1 and the ratio of total β -subunits ($\beta + \beta'$) to α -subunit was 4:1. The approximate molecular weight of the holoenzyme was calculated from its hydrodynamic properties (Table II). A Stokes radius of 88 \AA was measured by gel filtration and a sedimentation coefficient ($s_{20,w}$) of 14.0 S was measured by sucrose density gradient centrifugation. A molecular weight of 508,000 ($\pm 48,000$) was calculated from these parameters as described in Table II. The cerebellar isozyme consistently appeared smaller than the forebrain isozyme. This difference was observed even in parallel density gradients run at the same time. The subunit structure of the cerebellar holoenzyme that is most consistent with its molecular weight and the ratio of its subunits is a decamer of $\sim 8 \beta/\beta'$ -subunits and $\sim 2 \alpha$ -subunits. The molecular weight of such a holoenzyme would be 574,000, slightly higher than the range indicated by its hydrodynamic properties.

Comparison of the Subunits of Forebrain and Cerebellar Isozymes

Iodinated Peptide Maps—We compared maps of iodinated peptides of the α - and β -subunits of the cerebellar kinase to those of the forebrain kinase (Fig. 2). Maps of the β -subunits of the two isozymes were identical. Maps of the α -subunits, however, showed a few reproducible differences. Maps of the β' -subunits from both isozymes (not shown) reveal that they are closely related to the β -subunits. As has been noted before (28, 37), peptide maps of the α - and β -subunits of the forebrain

TABLE I
Summary of purification of rat cerebellar Type II Ca²⁺/calmodulin-dependent protein kinase

Step	Total activity ^a μmol/min	Total protein ^a mg	n	Specific activity ^b μmol/min/mg	Purification -fold	Recovery %
1. Homogenate	13.5 ± 1.5	2418 ± 450	5	0.006	1.0	100
2. 170,000 × g supernatant	10.4 ± 1.9	724 ± 110	5	0.013	2.8	77
3. DEAE-agarose pool	1.98 ± 0.3	175 ± 23	5	0.012	2.2	15
4. (NH ₄) ₂ SO ₄ ppt.	1.74 ± 0.3	83 ± 13	5	0.021	4.1	13
5. Sucrose gradient	1.38 ± 0.2	16 ± 4	3	0.091	16.8	10
6. Calmodulin-Sepharose pool	0.76 ± 0.2	0.49 ± 0.29	2	2.02	400	6

^a Values are mean ± S.D. of *n* experiments.

^b Average of *n* preparations.

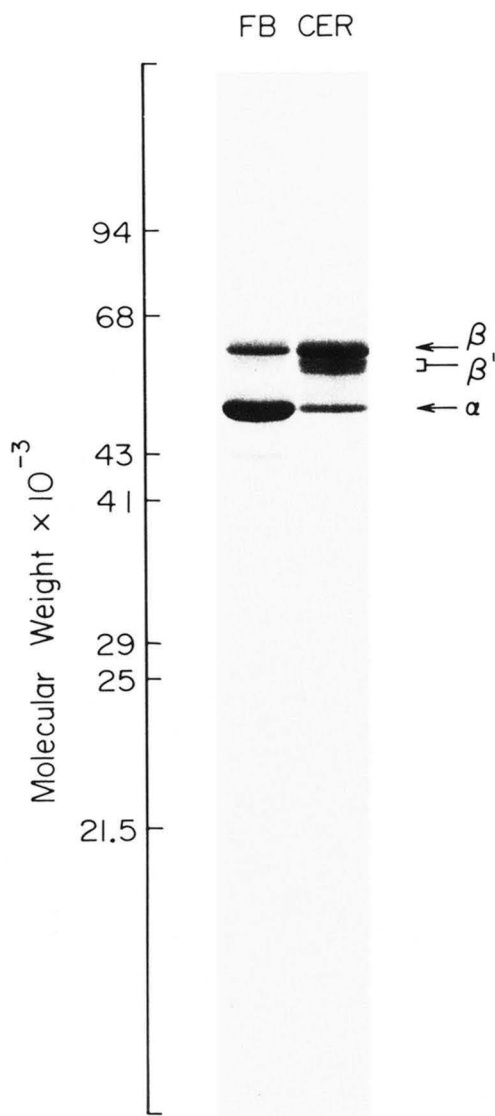


FIG. 1. Comparison of purified forebrain (FB) and cerebellar (CER) isozymes of rat brain Type II Ca²⁺/calmodulin-dependent protein kinase. Each isozyme (7.5 μg) was subjected to SDS-PAGE on a 12.5% gel, then stained with Coomassie Blue. Positions of molecular weight standards are indicated on the left.

kinase contain several peptides in common, indicating that they are structurally related. This is also the case for the cerebellar kinase. However, the structural homology between the α- and β-subunits is not as close as the homology between corresponding subunits in the two holoenzyme forms.

Immunochemical Comparison—Monoclonal and polyclonal

TABLE II

Physical properties of the forebrain and cerebellar forms of Type II Ca²⁺/calmodulin-dependent protein kinase

Property	Forebrain ^a	Cerebellum
Stokes radius (<i>a</i>)	94.7 ± 1.2 Å	88 ± 1.3 Å ^b
Sedimentation coefficient (<i>s</i> _{20,w})	16.4 ± 0.7 S	14.0 ± 1.1 S ^c
Frictional coefficient (<i>f</i> / <i>f</i> ₀)	1.67	1.67 ^d
Molecular weight (<i>M</i> _r)	650,000	508,000 ± 48,000 ^d
Holoenzyme composition	9 α:3 β/β' (654,000 Da)	2 α:8 β/β' (574,000 Da)

^a Values for the forebrain are those previously published by Bennett *et al.* (27).

^b Identical values were obtained in two experiments. The range is ± Stokes radii included in one column fraction.

^c Mean ± S.D. of three experiments.

^d Calculated according to the equation: $M_r = 6 \pi \eta_{20,w} s_{20,w} a N / (1 - \bar{\gamma} \rho_{20,w})$ where *N* = Avogadro's number, $\eta_{20,w}$ = viscosity of water at 20 °C, $\rho_{20,w}$ = density of water at 20 °C, and $\bar{\gamma}$ = partial specific volume, assumed to be 0.725 cm³/g. The frictional coefficient was determined by the formula: $f/f_0 = a(4\pi N/3\bar{\gamma}M_r)^{1/2}$.

antibodies which recognize specific subunits of the forebrain Type II CaM kinase on immunoblots were used to test the immunological relationship between the subunits of the forebrain and cerebellar isozymes. Two polyclonal rabbit antisera that recognize primarily the β-subunit of the forebrain kinase cross-reacted strongly with the cerebellar β/β'-subunits. A monoclonal antibody, 6G9, which binds to the α-subunit of the forebrain isozyme also bound to the α-subunit of the cerebellar isozyme (data not shown). Thus, the denatured subunits of the two kinases were indistinguishable by these immunochemical reagents.

Comparison of Enzymatic Properties of Forebrain and Cerebellar Kinases—The substrate specificity and catalytic constants of the forebrain and cerebellar isozymes were compared to determine whether their catalytic properties might differ significantly *in vivo*.

Substrate Specificity—The forebrain kinase phosphorylates a number of substrate proteins (27). We measured the ability of the cerebellar kinase to phosphorylate several of these same substrates (Table III). At this level of kinetic resolution, the substrate specificity of the two forms did not differ. Synapsin I and MAP₂ were phosphorylated at the highest rate.

Catalytic Constants—For each of the two kinases, we determined the apparent *K_m* values for synapsin I, MAP₂, and ATP, as well as the apparent *K_a* for calmodulin (Table IV). The apparent *K_m* values for synapsin I and MAP₂ were the same, however the *K_m* for ATP differed by a factor of ~1.8, the cerebellar kinase having the higher affinity. Since the intracellular ATP concentration is estimated to be about 1–2 mM (55), the difference between the two kinases is not likely to be significant *in vivo*. However, the cerebellar kinase also

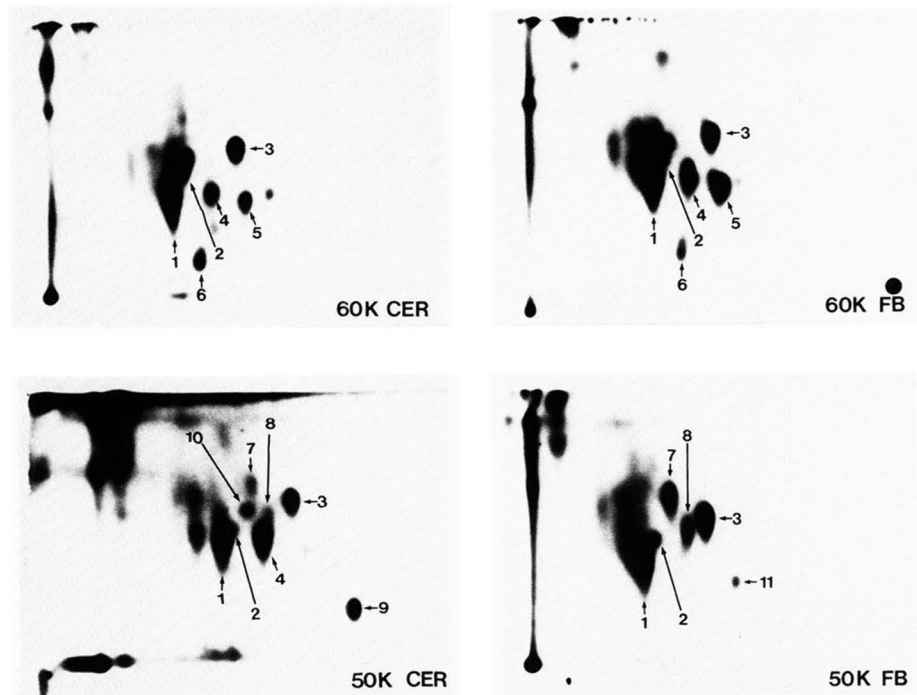


FIG. 2. Iodinated tryptic peptides of the α - and β -subunits of Type II CaM kinase isolated from forebrain (FB) and cerebellum (CER). Five μ g of pure kinase isolated from each brain region was subjected to SDS-PAGE and stained with Coomassie Blue. The bands corresponding to the α -, β -, and β' -subunits from each region were cut from the gel. Iodinated tryptic peptide maps were prepared as previously described (54). One μ l of the peptide solution was applied to the plate at the lower left. For electrophoresis, the anode was to the left and the cathode to the right for electrophoresis; chromatography was from bottom to top. The spots labeled 1, 2, and 3 were common to both subunits from both brain regions; 4, 5, and 6 were common to the β -subunits (4 was also found in the cerebellar α -subunit); 7 and 8 were common to the α -subunits (although differing in intensity); 9 and 10 were found only in the cerebellar α -subunit; and 11 is found only in the forebrain α -subunit. The identities of the various spots were confirmed by mapping of mixtures of peptides from the different subunits.

TABLE III

Substrate specificity of cerebellar Type II Ca²⁺/calmodulin-dependent protein kinase

Substrate	Concentration in assay mg/ml	Rate of Ca ²⁺ /calmodulin-stimulated phosphorylation	
		Cerebellum	Forebrain
		%	
Synapsin I ^a	0.1	100	100
MAP ₂ ^b	0.4	128	141
Histone ^c	0.2	21	14
Phosvitin	0.4	3	8
Casein	0.4	0	0
Phosphorylase b	0.4	2	3

^a The rate of Ca²⁺-stimulated phosphorylation of synapsin I was 33–37 pmol/min for the cerebellar kinase and 23–30 pmol/min for the forebrain kinase.

^b The source of MAP₂ was a microtubule protein preparation consisting of approximately 10% MAPs and 90% tubulin.

^c This sample of arginine-rich histones contained approximately 30% histone H3, which was the only protein phosphorylated. Calmodulin concentration in these assays was increased to 0.2 mg/ml.

had a consistently higher apparent affinity for Ca²⁺/calmodulin. The difference in affinities occurred in a range over which the concentration of the Ca²⁺/calmodulin complex would be expected to vary *in vivo*. Thus, this difference may have physiological significance.

Autophosphorylation of Kinase Subunits

The subunits of the Type II CaM kinase undergo rapid and stoichiometrically significant autophosphorylation under con-

TABLE IV

Comparison of K_m values of cerebellar and forebrain Type II CaM kinase isozymes

Substrate	Apparent K_m or K_a	
	Forebrain	Cerebellum
Synapsin I ^a	1.2 μ M \pm 0.1	1.1 μ M \pm 0.2
MAP ₂ ^b	130 nM	114 nM
ATP ^a	16 μ M \pm 1.3	9 μ M \pm 1.1
Calmodulin ^a	123 nM \pm 6	69 nM \pm 1

^a Values are the mean \pm S.D. of at least two experiments.

^b The K_m of the forebrain kinase for MAP₂ was determined by James Soha of Caltech. The K_m of the cerebellar kinase was determined in one experiment, using the same preparation of purified MAP₂.

ditions in which the kinase is active (27, 28). When crude brain homogenates are phosphorylated under conditions in which the kinase is maximally active, the most prominent endogenous phosphopeptides are the subunits of the kinase itself (27, 56). This probably reflects, at least in part, the relative abundance of the kinase in brain (35). We compared the Ca²⁺/calmodulin-stimulated endogenous phosphoproteins in forebrain and cerebellar homogenates to the autophosphorylated subunits of the Type II CaM kinase isozymes from each region (Fig. 3). The most prominent endogenous phosphopeptides in both homogenates were the kinase subunits. We confirmed this by comparison of phosphopeptide maps of the endogenous phosphoproteins and the phosphorylated subunits of the pure isozymes (data not shown). The patterns of phosphorylation shown in Fig. 3 were observed in fresh tissue

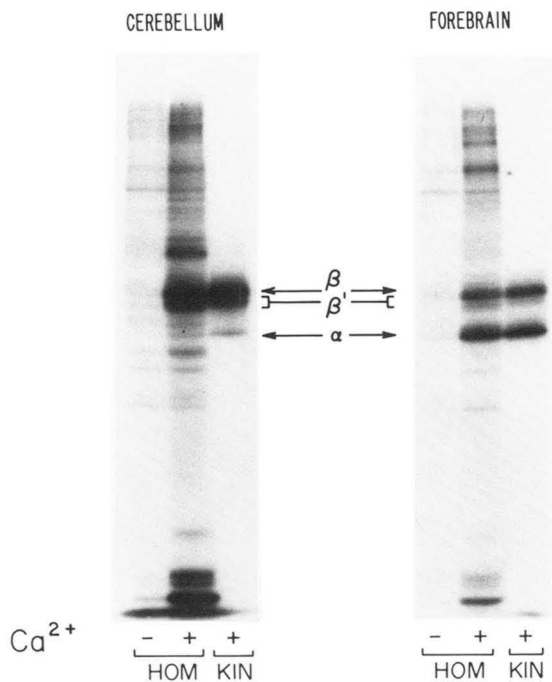


FIG. 3. Comparison of endogenous Ca^{2+} /calmodulin-dependent phosphorylation of crude brain homogenates and of pure Type II CaM kinases from forebrain and cerebellum. Samples of crude homogenates (HOM, 30 μg of forebrain, 120 μg of cerebellum) and pure kinase (KIN, 0.2 μg of forebrain and cerebellum) were incubated under phosphorylating conditions for 10 s with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described under "Experimental Procedures." The incubations were carried out in the presence or absence of Ca^{2+} as indicated. The samples were then subjected to SDS-PAGE and dried, and labeled bands were detected by autoradiography.

homogenates, indicating that the different proportions of kinase subunits were not generated artifactually during purification.

Concentration of Cerebellar and Forebrain Isozymes in Postsynaptic Density Fractions

The α -subunit of Type II CaM kinase is a major component of postsynaptic density fractions prepared from whole brain (36–38). However, several groups have reported that it is reduced in concentration or nearly absent in cerebellar PSDs (57–59; in these papers, the α -subunit is referred to as the 51K PSD protein or the major PSD protein). We wondered whether this reduction simply reflected the difference in subunit composition of forebrain and cerebellar Type II CaM kinase isozymes, so we examined the content of α - and β -subunits in PSD fractions from the two brain regions.

Endogenous Phosphorylation of PSDs—Freshly isolated PSDs from forebrain and cerebellum were labeled with ^{32}P by endogenous phosphorylation in the presence of calcium and calmodulin (Fig. 4). Under these conditions, the major endogenous phosphoproteins in the PSDs had the same molecular weights and were present in the same proportions as the subunits of the Type II CaM kinase isozyme from the corresponding region. However, the cerebellar PSD fraction appeared to contain reduced quantities of both subunits (note that the cerebellar PSD lane in Fig. 4 contains 5-fold more protein than the forebrain PSD lane).

Protein Composition of Cerebellar and Forebrain PSDs—We also compared Coomassie Blue-stained protein profiles of cerebellar and forebrain PSDs to the profiles of purified Type II CaM kinases from each region (Fig. 5). As was previously reported, the predominant protein in the forebrain PSDs was

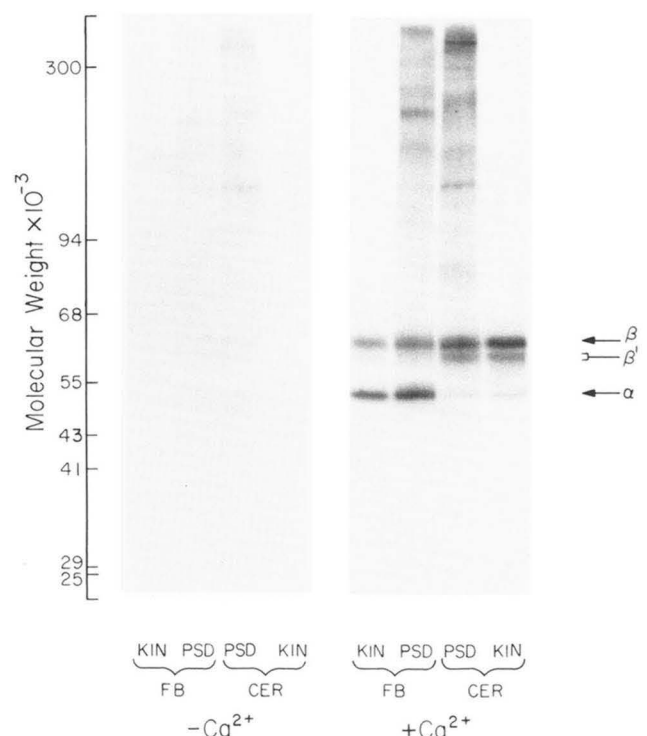


FIG. 4. Comparison of endogenous Ca^{2+} /calmodulin-dependent phosphorylation of PSDs and purified Type II CaM kinase from forebrain (FB) and cerebellum (CER). Samples of PSDs (5 μg from each region) and pure kinase (KIN) (0.6 μg from each region) were incubated under phosphorylating conditions for 10 s with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described under "Experimental Procedures." The incubations were carried out in the presence or absence of Ca^{2+} as indicated. Samples containing 60 ng of each pure kinase, 2.5 μg of the cerebellar PSD, and 0.5 μg of the forebrain PSD were subjected to SDS-PAGE and dried, and the labeled bands were detected by autoradiography.

the 50-kDa protein that is identical with the α -subunit of Type II CaM kinase. Protein bands corresponding to the β -subunits were also visible. In contrast, the cerebellar PSDs contained much less α -subunit; no increase in bands corresponding to the β -subunits was apparent. We measured the content of each of the subunits in the PSD fractions by a quantitative immunoblot method (35). A polyclonal antisera (Darcy) was used to quantitate the β -subunit and a monoclonal antibody (6G9) was used to quantitate the α -subunit (Table V). Fig. 6 illustrates the reactions of these antibodies with PSD fractions. The forebrain PSDs contained ~ 3.5 α -subunits for each β -subunit; together, the subunits comprised 16–18% of the total PSD protein. In contrast, the cerebellar PSDs contained ~ 4 β -subunits for each α -subunit and the subunits comprised only 1–2% of the total PSD protein. Thus, PSD fractions from each region contained the isozyme of the Type II CaM kinase that predominates in that region, but the concentration of the kinase in cerebellar PSDs was only about 8% of the concentration in forebrain PSDs. The kinase holoenzyme is about 1% of total forebrain protein and about 0.3% of total cerebellar protein (35). Therefore, the Type II CaM kinase is 20-fold enriched in forebrain PSDs and only 3–4-fold enriched in cerebellar PSDs.

DISCUSSION

We have shown that brain "Type II" Ca^{2+} /calmodulin-dependent protein kinase exists in a different isomeric form in cerebellum as compared to forebrain. The cerebellar isozyme contains 50- and 60-kDa α - and β -subunits that are

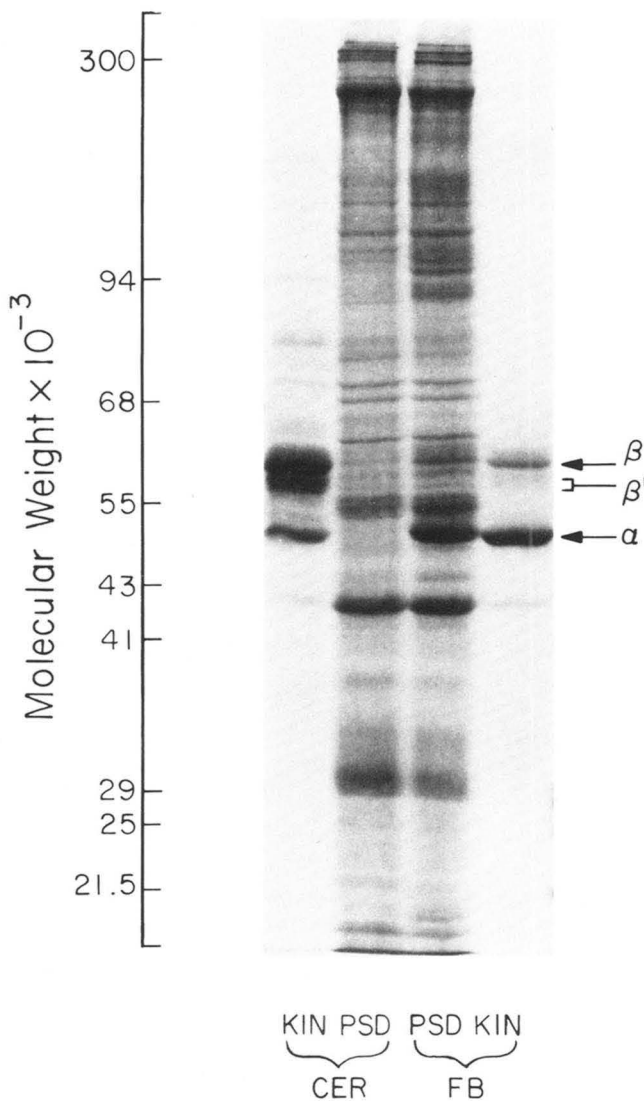


FIG. 5. Protein composition of PSD and Type II CaM kinase (KIN) isolated from either forebrain (FB) or cerebellum (CER). Six μ g of purified kinase and 40 μ g of PSD protein from each region were subjected to SDS-PAGE on a 10% gel then stained with Coomassie Blue. Positions of molecular weight standards are indicated on the left.

TABLE V

Concentration of α - and β -subunits of Type II CaM kinase in PSD fractions from forebrain and cerebellum

Region	α -Subunit/ PSD protein ^a	β -Subunit/ PSD protein ^a	Molar ratio (α : β)	Kinase holoenzyme as % PSD protein ^a
	ng/ μ g	ng/ μ g		%
Cerebellum	2.4 \pm 0.2 (8) ^b	10.5 \pm 1.1 (8) ^b	1.0:3.7	1.3 \pm 0.1
Forebrain	129.6 \pm 9.6 (35) ^b	44.5 \pm 2.7 (12) ^b	3.5:1.0	17.4 \pm 1.5

^a \pm S.E.M.

^b Number of determinations.

closely related to those in the forebrain; however, they are present in a different ratio. The cerebellar α : β ratio is 1:4, whereas the forebrain α : β ratio is 3:1. The resolution of our analytical methods is not sufficient to tell if the kinase holoenzymes from the two brain regions exist as unique oligomers that each contain the same number of α - and β -subunits. It

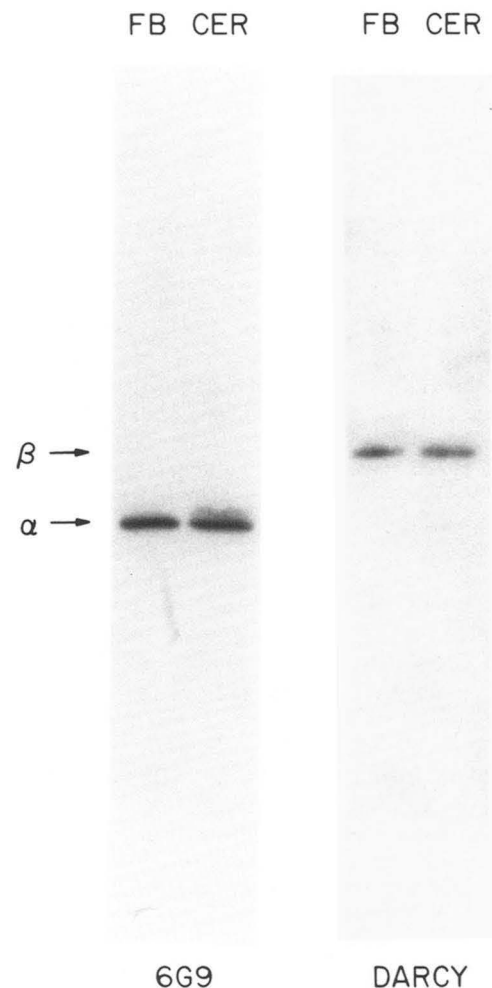


FIG. 6. Immunoblots of PSDs isolated from forebrain (FB) and cerebellum (CER). The left panel shows the reaction of a monoclonal antibody (6G9), which is specific for the α -subunit, with 0.43 μ g of forebrain PSDs and 15 μ g of cerebellar PSDs. The right panel shows the reaction of a polyclonal antisera (Darcy), which is specific for the β -subunit, with 2.3 μ g of forebrain PSDs and 7 μ g of cerebellar PSDs. Immunoreactive bands were detected as described under "Experimental Procedures."

is possible that we have measured an average molecular weight of heterogeneous oligomers that were assembled randomly from newly synthesized subunits. In the latter case, the difference in average holoenzyme composition between the two regions would simply reflect a difference in the ratio of subunits synthesized. Further studies will be necessary to determine whether intermediate isozyme forms exist in other brain regions (35).

The β -subunits from the two brain regions show extensive immunological cross-reactivity and have identical iodinated tryptic peptide maps. The α -subunits also show immunological cross-reactivity and their maps differ by only a few peptides. Studies by our laboratory and others have suggested that the α - and β -subunits are themselves structurally homologous and may both be catalytic (27, 37). The properties of the cerebellar isozyme are consistent with this notion. The specific activity of the purified cerebellar kinase is similar to that of the forebrain kinase, although the ratio of its subunits is dramatically different. In addition, the protein substrate specificities of the two isozymes do not differ, insofar as we have measured them.

What then is the functional significance, if any, of the

different kinase forms? There are several possibilities. In peripheral tissues, closely related isoenzymes sometimes differ in critical kinetic constants or in their association with specific subcellular organelles (60). The cerebellar kinase shows a consistent 2-fold higher apparent affinity for the Ca^{2+} /calmodulin than does the forebrain kinase. The difference is in a concentration range over which calcium-bound calmodulin is likely to vary *in vivo*. Thus, the cerebellar kinase may be more sensitive to increases in calcium concentration than the forebrain kinase.

A second possible functional difference between the α - and β -subunits is in their association with subcellular structures *in vivo*. Protein isozymes are known to have distinct subcellular locations both in peripheral and in neural tissue. This specific organization of the cytoplasm may be important for efficient cellular functioning (60). Recently, isozymes of various neuronal cytoskeletal proteins have been shown to have different subcellular locations. In chick cerebellum, β -spectrin is confined to the plasma membrane of the soma, while γ -spectrin (fodrin) is associated with the membranes of both the soma and processes (61). Similarly, MAP_2 is located in neuronal dendrites and perikarya, while MAP_1 is more evenly distributed throughout the neuron (62, 63). The results presented here suggest that the subcellular locations of the forebrain and cerebellar Type II CaM kinase isozymes differ. Although more of the cerebellar isozyme (85%) than of the forebrain isozyme (50%) is recovered in the particulate fraction of brain homogenates, cerebellar PSDs contain only ~8% as much Type II CaM kinase as do forebrain PSDs. This suggests that the α -subunit is involved in anchoring of the kinase within PSDs, while the β -subunit may have a higher affinity for different subcellular structures. It will be interesting to determine what other particular structures the cerebellar kinase is bound to.

In summary, we have confirmed and extended the observation made by several groups that the major difference in protein composition between cerebellar and forebrain postsynaptic density fractions is their content of a major 51-kDa protein (46, 58, 59). The 50-kDa protein is the α -subunit of the Type II CaM kinase (36–38). We have shown that PSD fractions from cerebellum contain reduced quantities of this protein because the cerebellar kinase contains less α -subunit and more β -subunit than the forebrain kinase and has a reduced affinity for the PSD fraction. This quantitative difference in the concentration of the Type II CaM kinase in PSDs from neurons in the two brain regions may produce different responses in these neurons to postsynaptic changes in calcium flux.

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